Differences among heat-treated, raw, and commercial peanut extracts by skin testing and immunoblotting

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Background: Peanut allergenicity has been reported to be influenced by heat treatment, yet the commonly available extracts for skin prick testing (SPT) are derived from raw extracts.

Objective: To assess the effect of heat treatment on the SPT reactivity and specific IgE binding to peanut.

Methods: Three commercial extracts and 3 laboratory-prepared extracts, including raw, roasted, and boiled, were used for SPT in 19 patients with suspected peanut allergy and in 4 individuals who eat peanut without any symptoms. Serum samples were obtained to measure total IgE in addition to specific IgE binding to the study extracts by immunoblotting. Peanut allergy was confirmed with challenge test unless the individual had a convincing history of a severe reaction.

Results: Eleven study participants were considered peanut allergic based on a strong history or positive challenge test result. SPT with the prepared and commercial reagents showed that the boiled extract had the highest specificity (67% vs 42%–63% for the other extracts). The prepared extracts showed similar SPT sensitivity (81%). Three patients with a history of severe reaction and elevated specific IgE levels to peanut to the 3 study extracts had variable SPT reactivity to 1 or more of the commercial extracts. IgE binding to Ara h 2 was found in nearly all patients, regardless of their clinical reactivity.

Conclusions: None of the extracts tested showed optimal diagnostic reliability regarding both sensitivity and specificity. Perhaps testing should be performed with multiple individual extracts prepared by different methods.

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INTRODUCTION

Peanut allergy is often severe and lifelong.[1] Patients may react to as low as 10 mg of peanut,[2] and complete avoidance can be difficult because of cross-contamination or hidden sources of peanut in foods.[3] Some patients may even react by skin contact[4] or smell.[5] For a food allergic individual, identifying the offending food may be difficult because of the inability to recognize the type of "nuts" and variability in food allergy tests.[6]

The major skin peanut allergens have been identified as Ara h 1, Ara h 2, Ara h 3/4, and Ara h 6.[7–11] Currently, there is no commercially available test that differentiates the specific IgE (sIgE) binding to each of these proteins; however, IgE binding to specific allergen components have shown some increased specificity in food allergic individuals.[12] It is possible to determine sIgE binding to the major peanut allergens, which may be more useful than the cumulative sIgE in providing prognostic information.[13–15]

In addition to the medical history, screening for food allergy includes skin prick tests (SPTs) or sIgE measurement by enzyme-linked immunosorbent assay. Several studies have attempted to establish reliable diagnostic cutoff values for these tests, but reported values varied widely for both SPT[16–19] and sIgE measurement,[20–26] with suboptimal correlation with food challenge testing. Even when suggested threshold values for sIgE are used to predict clinical peanut allergy, there is still wide variability in the criteria used to confirm a diagnosis of peanut allergy.[27,28]

Rance et al[25] found a positive correlation between positive SPT results and food challenges (approximately 80%) when raw egg, cow's milk, and peanut were used in contrast to commercial extracts, which correlated only 50% of the time. This suggests that skin testing with raw food provides higher sensitivity.[25,26,29]

An added complexity of food allergy testing is the preparation of extracts from differently processed foods. One study using raw, roasted, and commercial peanut extracts reported that the SPT with roasted peanut had the best correlation with a clinical history, but this was not verified by oral challenge.[25] Others have reported differences in sIgE binding between roasted and raw peanut.[30–33] In this study, we were interested in SPT and sIgE reactivity of peanut extracts prepared by

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different processing methods compared with commercially available extracts.

METHODS

Study Participants

Nineteen individuals with suspected peanut allergy were compared with 4 peanut-tolerant controls (eating peanut freely without any symptoms). A detailed history and physical examination were performed on each study participant, then SPT and oral challenge were performed. A blood sample was also obtained. The study protocol was approved by the institutional review board, and a written informed consent or ascent was obtained from all participants.

Commercial Reagents

Skin prick testing extracts were obtained from ALK-Abello (Roundrock, Texas), Greer Laboratories (Lenoir, North Carolina), and Hollister-Stier Laboratories (Spokane, Washington). According to the manufacturers (oral communication, February 2009), these extracts are prepared from raw peanut varieties, including Virginia and Spanish peanuts.

Prepared Reagents

Raw, roasted (160°C for 5 minutes), or boiled (100°C for 5 minutes) peanuts were ground into a paste and defatted with petroleum ether extraction. Two grams of each was solubilized, then 10 mL of phosphate-buffered saline (PBS), pH 8, was added and sonicated 5 times (1 minute each) and centrifuged at 11,780*g* for 15 minutes. The supernatant was filter-sterilized. Protein concentrations were prepared and commercial extracts were determined with protein assay reagent (Bio-Rad, Hercules, California). Concentrations for each of the study extracts were normalized with that of the commercial extracts. Autoclaved glycerol and phenol were mixed with the prepared extracts (study extracts) to match the commercial extracts, all in 50% glycerol and 0.4% phenol.

Skin Prick Testing

Accuset SPT lancet (ALK-Abello), a dual-pronged, disposable SPT device, was used to perform SPTs. The patients underwent SPTs with 3 commercial (ALK, Hollister-Stier, and Greer) and 3 study (raw, roasted, and boiled) extracts. Histamine and negative control (saline) SPTs were included. For uniformity, SPTs were performed by the same physician, and the results read after 20 minutes and graded as follows: 0, similar to negative controls (no wheal or erythema); 1+, larger than the negative controls by 25% to 50% (<3 mm); 2+, larger than the negative control by 50% to 75%, with a wheal of 3 to 5 mm; 3+, similar to histamine wheal without pseudopods (5–7 mm); 4+, larger than histamine by 25% to 50% without pseudopods; and more than 4+, larger than histamine by more than 50% with pseudopods.

Food Challenge Testing

Patients underwent titrated oral peanut challenge, supervised in the clinic as previously described,[34] unless there was a strong medical history with a positive SPT result. A total of

Table 1. Total IgE, Peanut Specific IgE (sIgE), and Skin Prick Test (SPT) Results in 11 Peanut Allergic Individuals

Patient No./ sex/age	sIgE level, IU/mL	Total IgE, IU/mL	SPT results						Atopic history	Peanut challenge	Other foods suspected
			Greer	Hollister-Stier	ALK-Abello	Raw	Boiled	Roasted			
1/M/14 mo	0.12	12	NT	NT	0	0	0	0	E to milk and egg, never had peanuts	N, oral P, V for 2 hours	Egg, milk
2/M/12 y	0.23	260	3+	3+	3+	3+	0	4+	AN, AR, AS, SOB to peanut	NT	None
3/M/4 y	0.45	71	NT	NT	3+	4+	3+	4+	AN, AE lips/tongue, U	NT	None
4/F/6 y	0.52	742	>4+	>4+	0	1+	2+	>4+	AE, V to Milk and shrimp	Severe AE	Egg, shrimp
5/F/7 y	2.36	126	0	2+	4+	4+	>4+	>4+	AN to peanut, V to shrimp	NT	Shrimp
6/M/2.5 y	3.42	62	NT	NT	4+	>4+	>4+	3+	E to peanut	NT	None
7/F/9 y	6.09	1203	2+	3+	0	2+	2+	0	AN, U, AE lips/ tongue/face, SOB to peanut E, AR to wheat, tomato and orange, AS, AN to strawberry	NT	None
8/M/4 y	8.05	1639	>4+	3+	>4+	4+	>4+	>4+	U, AE to peanut, U with SPT	NT	Egg, milk
9/M/9 y	8.36	566	>4+	4+	4+	0	3+	>4+	AN to peanut, U to shrimp and egg	NT	Egg, shrimp
10/M/20 mo	8.55	270	NT	NT	4+	4+	>4+	>4+	E to peanut, milk, shrimp and fish	V	Milk, egg, shrimp
11/M/9 y	39.3	124	>4+	>4+	3+	3+	3+	3+	U to peanut at 15 mo	N, oral P, V for 2 hours	None

Abbreviations: AE, angioedema; AS, asthma; AN, anaphylaxis; E, eczema; N, nausea; NT, not tested; P, pruritus; SOB, shortness of breath; U, urticaria; V, vomiting.

7 were not subjected to oral challenge testing; 6 (# 2, 3, 5, 7, 8, 9) because of a convincing history of severe reactions and 1 (# 6) because of parental refusal. The challenge was performed single-blind or openly because the claimed reactions were all objective and most of the study participants were young children.

Total and sIgE Determinations

The total serum IgE level was measured by chemiluminescent enzyme-linked immunosorbent assay (IBT Laboratories, Lenexa, Kansas), and peanut sIgE levels were measured using the UniCap system (Pharmacia Diagnostics, Uppsala, Sweden).

SDS-PAGE and Western Blot Analysis

The total protein content of each of the sixth extracts was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), stained with Gel-Code Blue stain (Pierce, Rockford, Illinois), and photographed. For individual allergen Western blot analysis, the samples were subjected to SDS-PAGE and transferred to a polyvinylidene fluoride membrane, blocked for 1 hour in 5% Botto (5% nonfat, dry milk in PBS containing 0.5% Tween [PBST]), and incubated for 1 hour with serum IgE (1:10 in PBST) or specifically prepared (Sigma Immunosys, The Woodlands, Texas) anti-Ara h 1, Ara h 2, or Ara h 3 (1:5,000, in Blotto). The membranes were then washed and incubated with the relevant horseradish peroxidase–labeled secondary antibody (anti-chicken IgY) (Sigma Chemical Company, St. Louis, Missouri) at 1:100,000 or anti-human IgE 1:10,000 in 2% blotto for 30 minutes. The membrane was developed with ECL-Plus Western substrate (Amersham Bioscience Corp, Piscataway, New Jersey) and the signal visualized using a CCD camera system (Fuji Photo Film Co Ltd, Duluth, Georgia).

RESULTS

Study Participant Characteristics and Oral Challenge Responses

Of the 19 study participants with suspected peanut allergy, 9 had a history of urticaria, 7 had angioedema, 7 had exacerbation of eczema, 2 had vomiting, 5 had wheeze or shortness of breath, 2 had asthma exacerbation, 3 had rhinorrhea, and 1 had contact urticaria. Seven participants were not tested by challenge, the other 12 were challenged and 5 reacted (Table 1).

Skin Test Reactivity to Prepared and Commercial Extracts

Significant variability in SPT reactivity to commercial and study extracts was observed. For example, 1 participant who strongly reacted to the 3 study extracts and 2 commercial ones (ALK and Hollister-Stier) had a negative SPT result to the Greer extract (Table 1). Another participant reacted to the 3 study extracts and 2 commercial extracts (Hollister-Stier and Greer) but not to the ALK extract, whereas 4 other participants did not react to any of the study extracts. Eight of the patients who were sensitized (positive SPT or sIgE test results) (Table 2) were able to tolerate peanut when challenged. Most peanut-tolerant patients who had a positive SPT result to multiple test extracts had high total IgE and/or sIgE levels of class 2 or greater. For example, 1 patient had a total IgE of 15,117 kU/L and a 2+ positive SPT result to all 3 commercial extracts (Table 2). Also, 19 of the 23 total study participants had a positive SPT result to at least 1 of the study

Table 2. Total IgE, Peanut Specific IgE (sIgE), and Skin Prick Test (SPT) Results in 12 Peanut-Tolerant Individuals

Patient No./ sex/age	sIgE level IU/mL	Total IgE, IU/mL	SPT results						Atopic history	Food suspected
			Greer	Hollister-Stier	ALK-Abello	Raw	Boiled	Roasted		
1/M/5 y	9.07	565	0	0	4+	0	0	4+	W, E to peanut	None
2/F/2 y	35.5	453	NT	NT	>4+	3+	3+	3+	U, AE	Egg
3/M/4 y	2.39	630	NT	NT	0	0	0	0	AR, E to peanut, and soy	Soy
4/M/2 y	0.85	189	NT	NT	2+	0	0	2+	E to milk egg, peanut	Milk, egg
5/M/23 y	0.39	15,117	2+	2+	2+	0	0	0	E to milk, pecan, shrimp and peanut	Milk, egg
6/F/24 y	0	421	2+	3+	3+	2+	0	0	AN to shrimp, AE of mouth to peanut	Shrimp
7/F/16 mo	5.76	191	>4+	3+	>4+	4+	4+	>4+	U, W to peanut AE, U, V to milk	Milk
8/M/2 y	0.13	441	NT	NT	3+	3+	3+	2+	E to egg and peanut	Egg
9/F/14 y	0	25	0	0	0	0	0	3+	U	None
10/F/4 y	0		0	0	0	0	0	0	W, U to shrimp	Milk, beef pork, shrimp
11/F/39 y	0	343	0	0	0	0	0	0	AE tongue/lips/eyes/face to shrimp	Shrimp
12/M/15 y	0	126	4+	4+	0	4+	0	0	GI upset to milk	None

Abbreviations: AE, angioedema; AN, anaphylaxis; E, eczema; GI, gastrointestinal; NT, not tested; U, urticaria; V, vomiting; W, wheezing.

extracts, which had similarly variable results as the commercial extracts in our total group. Of the 19 study participants, 12 (63%) had a larger or equal SPT reaction to roasted peanut compared with the other 2 study extracts (raw and boiled).

Protein and Allergen Profiles of Each Extract

The SDS-PAGE of the peanut extracts showed differences in protein profiles of the 6 extracts used in this study (Figure 1A). Smears appeared at the top of the boiled, roasted, Hollister-Stier, and Greer extracts, which indicates the oligomerization of proteins due to heat treatment or storage. The Ara h 1 monomer (63 kDa) and a known breakdown product (approximately 36 kDa) appeared in all extracts when using anti–Ara h 1 antibody in a Western blot (Figure 1B). Distinct oligomeric forms (as opposed to smears) of Ara h 1 were most apparent in the boiled (lane 3) and roasted (lane 4) extracts at approximately 130 (dimer) and 190 kDa (trimer) and higher oligomeric forms consisting of combinations of dimers and trimers (molecular weight >250 kDa). The anti–Ara h 1 antibody is also recognizing smears in the raw extracts that are not seen in the SDS-PAGE. These higher oligomeric forms of Ara h 1 did not appear in the commercial extracts. Ara h 2 doublet bands are seen at 18 and 21 kDa (Figure 1C). Ara h 6, a band at approximately 17 kDa, was observed, which is recognized by the anti–Ara h 2 antibody due to 52% sequence identity. Detection of a fourth lower band is Ara h 7, which has sequence identity to Ara h 2 (36%) and Ara h 6 (31%). Ara h 2 levels were more consistent among the different extracts than the other allergens with the highest level in the extract from Greer (lane 7). The Hollister-Stier extract had the lowest Ara h 2 and Ara h 6 levels. Ara h 3 acidic subunits appear as doublet bands of 36 and 40 kDa in Figure 1. Two Ara h 3 lower-molecular-weight bands at 25 and 29 kDa are also recognized by this antibody, which was made against the 40-kDa acidic subunits of Ara h 3 (Figure 1D). A very low level of Ara h 3 was detected in the extract from Greer (lane 7) and somewhat low levels in the roasted (lane 4) extract and oligomeric species (appearing as smears) recognized by anti–Ara h 3 antibody in the Hollister-Stier extract (lane 6).

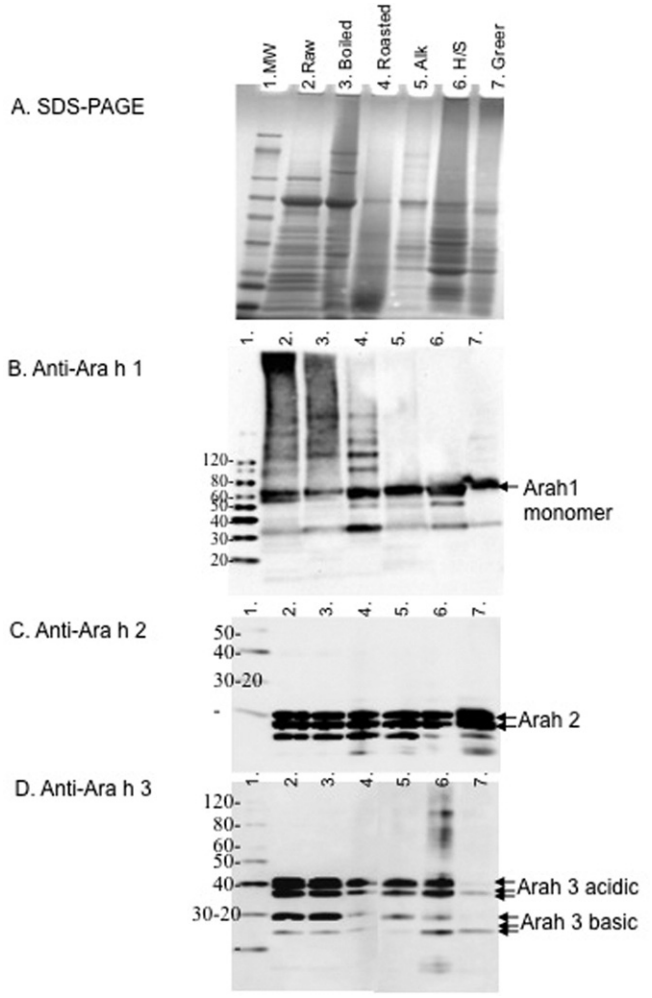

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis performed on skin prick test extracts. SDS-PAGE (A) and Western blot analysis were performed using anti–Ara h 1 (B), anti–Ara h 2 and 6 (C), and anti–Ara h 3 (D) antibodies. The lanes in all panels (A-D) are in the same order: molecular weight (MW, 1), raw (2), boiled (3), roasted (4), ALK-Abello (ALK, 5), Hollister-Stier (HS, 6), and Greer (G, 7).

Comparison of SPT and IgE Binding Results in Allergic and Nonallergic Study Participants

In Figure 2, representative IgE binding profiles of peanut allergic (left panels) and nonallergic individuals (right panels) are shown with a score representation of the SPT reaction (from 0 to 4+) to each extract. In serum samples of peanut allergic patients (Figure 2, left panels), IgE binding to the raw and boiled extracts (lanes 2 and 3) show that IgE antibodies that recognize Ara h 1, Ara h 2, Ara h 3/4, and Ara h 6 were present in all 5 patients' serum samples. In general, the weakest IgE binding was seen to the roasted and Hollister-Stier extracts, both of which resulted in the largest wheal size diameters in SPT. Although the major peanut allergens (Ara h 1, Ara h 2, Ara h 3, and Ara h 6) were present in all extracts (Figure 1), the serum IgE of individual patients only recognized these allergens in some of the extracts.

Western blots were also performed on 2 serum samples from 2 peanut-tolerant individuals (patients 10 and 11). Three study participants (patients 1, 5, and 8) were suspected of peanut allergy but did not react to challenge (Figure 2, right panels). Four of these peanut-tolerant participants (patients 1, 8, 10, and 11) showed preferential binding to Ara h 2 and all showed binding to Ara h 6. IgE binding intensity differed among the study participants but tended to be greatest to the raw and boiled study extracts, minimal to the roasted extract, and variable in the commercial extracts (Figure 2). It was noted that the binding to the major allergens Ara h 2, Ara h

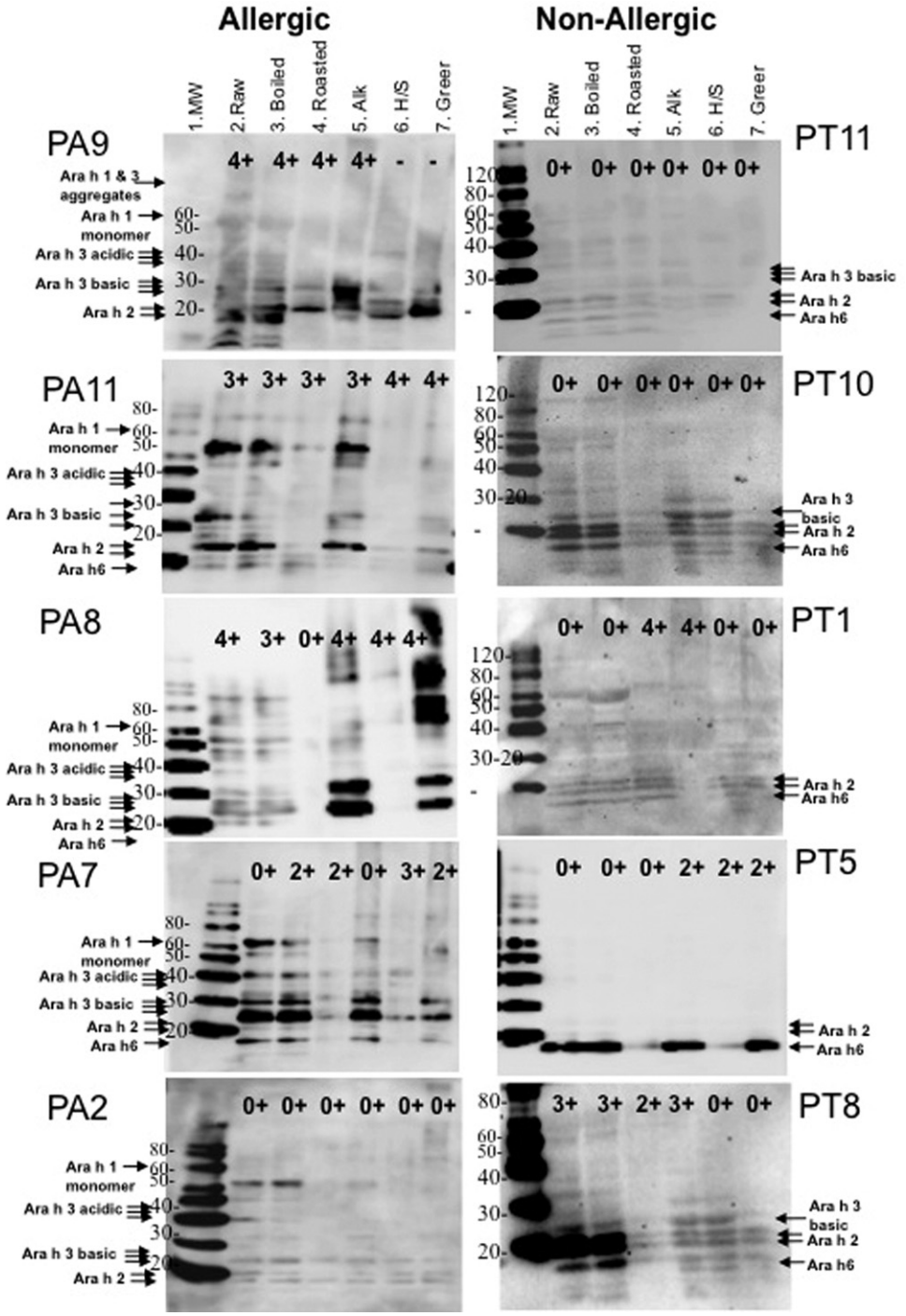

Figure 2. Western blot analysis of skin prick test (SPT) extracts using serum IgE of patients. Peanut allergic patients are shown on the left; nonallergic controls, 3 (patients 8, 10, and 11) never suspected and 2 (patients 1 and 5) with a suspected history of peanut allergy that tolerated oral challenge to peanut, are on the right. The lanes on all 10 membranes are in the same order and indicated in the figure. The numbers (0+, 2+, 3+, 4+) indicate the SPT scores; N indicates not tested.

6, and/or the 23-kDa basic subunit of Ara h 3 is distinctly stronger for all of the nonallergic patients than to any of the other allergens, with no particular correlation to sIgE or total IgE levels (Table 2).

Sensitivity and Specificity of SPT Reagents

The sensitivity and specificity of the various SPT reagents were calculated in this limited group (Table 3). SPTs showed that the boiled extract had the highest specificity (67% vs

Table 3. Sensitivity and Specificity of Peanut Skin Prick Test Reagents

Extract	Allergic, SPT positive, No. (% sensitivity)	Allergic, SPT negative, No. (% false negative)	Nonallergic, SPT positive, No. (% false positive)	Nonallergic, SPT negative, No. (% specificity)
Greer	6/7 (85)	0/7 (0)	4/8 (50)	4/8 (50)
Hollister-Stier	7/7 (100)	1/7 (14)	3/8 (38)	5/8 (63)
ALK-Abello	8/11 (67)	3/11 (27)	7/12 (58)	5/12 (42)
Raw	9/11 (81)	2/11 (18)	5/12 (42)	7/12 (58)
Boiled	9/11 (81)	2/11 (18)	4/12 (33)	8/12 (67)
Roasted	9/11 (81)	2/11 (18)	6/12 (50)	6/12 (50)

42%–63% for the other extracts). The sensitivity of the prepared extracts was the same (81%). Although Hollister-Stier had the highest sensitivity, the number of patients tested with the Hollister-Stier and Greer extracts were much fewer (15 vs 23 patients). There was significant variability in the sensitivity and specificity of the commercial extracts.

DISCUSSION

Evaluation of IgE sensitization by SPT and sIgE measurement are the most common allergy screening procedures used in practice. The reliability of these tests as clinical predictors is not optimal because these results often do not correlate with clinical food allergy symptoms. In this study, the diversity in the total protein and major allergens, Ara h 1, Ara h 2, Ara h 3, and Ara h 6, in each of the 6 extracts was tested to determine whether protein profiles or the allergen recognition pattern of a patient's IgE could potentially explain variability in SPT and sIgE tests. We found that the major allergens, Ara h 1, Ara h 2, and Ara h 3/4, were present in all the extracts but with a variable protein profile and allergen content. Interestingly, the patients' IgE could bind to individual allergens in the extracts to various degrees, which indicates that even though the allergens are present within each extract, they are not recognized equally by serum IgE of the patient. This finding suggests that the extract preparation methods may be altering the IgE binding to individual allergens. The 3 study extracts were prepared from differently processed peanuts (raw, boiled, and roasted) but prepared in exactly the same way. Meanwhile, the commercial extracts were all derived from raw peanuts, but they were prepared by different companies, most likely using different methods that were not revealed. One recent study claimed that there was no difference between SPT results using prepared raw and roasted peanut extracts and commercial extracts from Hollister-Stier,[35] but it was not clear how the SPT material was prepared and applied. Another study has shown that although the protein profiles of commercial peanut extracts varied considerably, the SPT reactivity to these extracts did not.[36] In our study, a significant discrepancy is seen in SPT reactivity and sIgE binding to the different extracts.

Different allergenic proteins in peanut vary in their sensitizing capacity, and food processing can change the degree of allergenicity of various food proteins.[31] It was previously found that roasted peanuts bind higher levels of IgE than raw peanuts.[32] In the current study, it is consistent that most peanut allergic patients had either equal or higher SPT reactivity to the roasted peanut extracts than to the boiled or raw extracts. Similar studies have shown that patients react differently to raw vs cooked fish, boiled vs freeze-dried raw egg white, and fresh vs commercial extracts of fruits and vegetables and nuts.[37,38] The method of food allergen extract preparation and storage alters SPT reactivity and makes a strong argument for more consideration of optimization and standardization of reagent preparation with foods.

The patients in our study all had IgE antibodies to Ara h 2 and Ara h 6. This may indicate that either 1 or more of the epitopes on these particular allergens are clinically irrelevant and may contribute to the high rate of false-positive SPT and sIgE test results with peanut extracts. We also found that, even though the highest Ara h 2 binding by the serum samples of 8 peanut-tolerant patients was often to raw and boiled peanuts, the boiled extracts had the lowest level of false-positive SPT results.

In this study, although raw, boiled, and roasted study extracts have the exact same SPT sensitivity (81%), the boiled and roasted peanut extracts have the higher specificity. Kemp et al[30] showed similar finding with roasted extracts, suggesting that heat-treated extracts may be more specific to detect patients with food allergy. Our study also shows that peanut allergic patients may go undetected if only 1 extract is used for SPT or in sIgE assay. If our findings are reproduced by more studies on larger number of patients, the information would be useful in preparing testing reagents of higher diagnostic reliability. Perhaps testing may need to be performed with multiple individual extracts or with a mixture of extracts prepared by different methods. The effect would be especially remarkable for peanut allergy because of its severity and high fatality rate.

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